СТРУКТУРНО-ФУНКЦИОНАЛЬНЫЙ АНАЛИЗ
БИОПОЛИМЕРОВ И ИХ КОМПЛЕКСОВ

UDC 577.15.577.151

CREATINE ETHYL ESTER: A NEW SUBSTRATE FOR CREATINE KINASE

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The creatine kinase/phosphocreatine system plays a key role in cell energy buffering and transport, particularly in cells with high or fluctuating energy requirements, like neurons, i.e. it participates in the energetic metabolism of the brain. Creatine depletion causes several nervous system diseases, alleviated by phosphagen supplementation. Often, the supplementation contains both creatine and creatine ethyl ester, known to improve the effect of creatine through an unknown mechanism. In this work we showed that purified creatine kinase is able to phosphorilate the creatine ethyl ester. The K_m and V_{max} values, as well as temperature and pH optima were determined. Conversion of the creatine ethyl ester into its phosphorylated derivative, sheds light on the role of the creatine ethyl ester as an energy source in supplementation for selected individuals.

ATP is the primary chemical energy source for the cell bioenergetics, but other substances participate in buffering the intracellular energy stores [1]. This buffering is achieved by the phosphagen kinase systems that consist of a specific kinase and its cognate phosphagen, which function as a large pool of 'high-energy phosphates' utilized to replenish ATP during periods of high energetic demand. In vertebrate tissues, the only phosphagen is phosphocreatine (PCr), and the corresponding phosphotransferase is creatine phosphokinase (CK). Creatine (Cr, *N*-aminoiminomethyl-*N*-methylglycine) is synthesized from the amino acids arginine, glycine and methionine [2]. Cr is produced endogenously by the liver, kidney, pancreas, and to some extent by the brain [3, 4]. Besides, it can be acquired exogenously through diet, as it is contained in fresh meat and fish [5]. Once transported into the cells, Cr is transformed into phosphocreatine (PCr), by CK, through a reversible transphosphorylation reaction, which involves ATP hydrolysis [6]. Almost all of the Cr in the body is located in the skeletal muscle in either the free (Cr: approximately 40%) or the phosphorylated (PCr: approximately 60%) form. The CK/PCr system plays an integral role in energy buffering and overall cellular bioenergetics [7]. In fact, the reaction is reversible, so that ATP can be generated from PCr, basing on the cellular energetic necessities. CK is a ubiquitous enzyme, located at strategic intracellular sites so as to couple the areas of high energy expenditure to those of efficient regeneration of ATP [8]. Several isoforms have been described: one ubiquitous, one sarcomeric, two mitochondrial (mtCK), and three cytosolic, whose amino acid sequence is consid-

erably conserved [9, 10]. Whereas MM-CK is expressed in the skeletal and cardiac muscle, MB-CK is exclusive of the cardiac muscle [11], and BB-CK is expressed in smooth muscle and in most non-muscle tissues [12, 13]. MtCK and cytosolic CK are linked in a so-called PCr/Cr-shuttle [14, 15]. PCr generated by mtCK in mitochondria is shuttled to the cytosolic CK that is intimately associated with the ATPases [16]. Therefore, by the conversion of PCr to Cr it is possible to recharge the cellular ATP pool for ATPase linked processes. Thus the role of PCr would also be as a "portable" form of cellular chemical energy that can be exchanged between subcellular ATP production sites (mitochondria and cytosol) and those of energy utilization (ATPases), forming a functionally coupled micro-compartment [17].

Due to this energetic capacity, Cr is part of dietary supplements for athletes, since it can improve athletic performance and muscle development [18, 19]. In fact, Cr has been shown to enhance performance in certain contexts with few adverse effects. In the athlete supplements another compound derived from Cr, i.e. the creatine ethyl ester (CEE), is often present. It has been suggested that the bioavailability of creatine is improved when CEE is ingested instead of creatine [20]. However, the role of CEE is poorly understood. It is only known that, inside the body, CEE can be converted to Cr, increasing the level of the phosphagen.

In the present paper we studied CK activity using CEE as a substrate and determined the kinetic parameters of this reaction. Data is discussed according to the hypothesis that the conversion of CEE into its phosphorylated form also occurs *in vivo*.

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EXPERIMENTAL

Creatine kinase assay, using creatine ethyl ester as substrate. Enzyme activity was measured by the enzyme coupling method of Bergmeyer [21] using the pyruvate kinase/lactate dehydrogenase system in which hydrolysis of ATP is coupled to the oxidation of NADH. The reaction mixture contained: 100 mM Tris HCl, pH 7.5, 2 mM $MgCl_2$, 75 mM KCl, 2 mM ATP, 0.5 mM phosphoenolpyruvate, 0.16 mM NADH, 10 IU of piruvate kinase and lactate dehydrogenase mix, 5 IU of creatine kinase (CK), in a final volume of 1 ml. The reaction was started by addition 50 mM of creatine ethyl ester (CEE), creatine (Cr), or creatinine. NADH oxidation was monitored spectrophotometrically by the decrease in absorbance at 340 nm ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}).

Kinetic studies. For kinetic studies, the CK assay was carried out varying the concentration of CEE (10−50 mM). The results were analyzed by a double reciprocal Lineweaver−Burk plot. K_m and V_{max} were computed from these plots. The same experiments were performed with Cr.

The effect of pH and temperature on enzymatic activity. The CK activity on CEE was measured in a pH range from 6 to 9, using 100 mM HEPES buffer. The effect of temperature on enzyme activity was investigated in the temperature range 20−75°C. Reaction temperatures were adjusted in a temperature-controlled water circulation bath.

RESULTS

The course of CK reaction was linear with time when CEE was used as a substrate (fig. 1a). In the presence of Cr (the standard CK substrate) enzyme activity was about six folds higher than with CEE as a substrate. CK activity was assayed also in the presence of creatinine, the heterocyclic derivate of Cr, and in this case the activity was undetectable (fig. 1b).

For kinetic analysis, CK assay was performed at various CEE concentrations. K_m and V_{max} values were calculated using the double reciprocal Lineweaver−Burk plot. Fig. 2a, shows the rate of ATP hydrolysis as a function of the CEE concentration (10−50 mM). In fig. 2b the Lineweaver−Burk plot indicates that the K_m value for CEE was 13.9 ± 2.1 mM, while Vmax value was 2.1 ± 0.3 μmol hydrolyzed ATP formed/min. Moreover, under the same assay conditions, K_m for Cr was 7 ± 0.9 mM and Vmax was 4.7 ± 0.7 μmol hydrolyzed ATP/min.

Study of the pH dependence of CK activity with CEE as a substrate in the pH range between 6 and 9 demonstrated the existence of an optimum at pH 7.5 (fig. 3). At pH 8, the CK activity is similar to the value observed at the optimal pH, while it decreases by about 50% when the pH is increased or decreased by 0.5, and by about 75% below pH 7 or above pH 8.5. The tem-
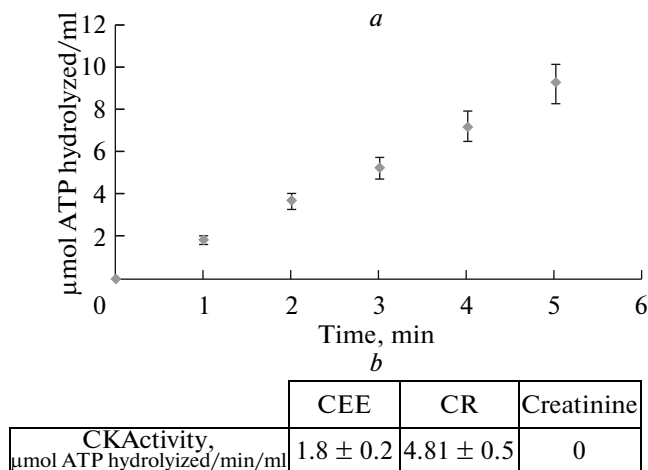

	CEE	CR	Creatinine
CKActivity, μmol ATP hydrolyized/min/ml	1.8 ± 0.2	4.81 ± 0.5	0

Fig. 1. *a* − The time course of the creatine kinase activity (IU/ml of volume assay) in the presence of 50 mM creatine ethyl ester. Each point represents the mean ± SD of six different experiments. *b* − Comparison of the CK activity assayed in the presence of creatine ethyl ester, creatine and cratinine. The activity in the presence of creatine ethyl ester is about 63% less than in the presence of creatine.
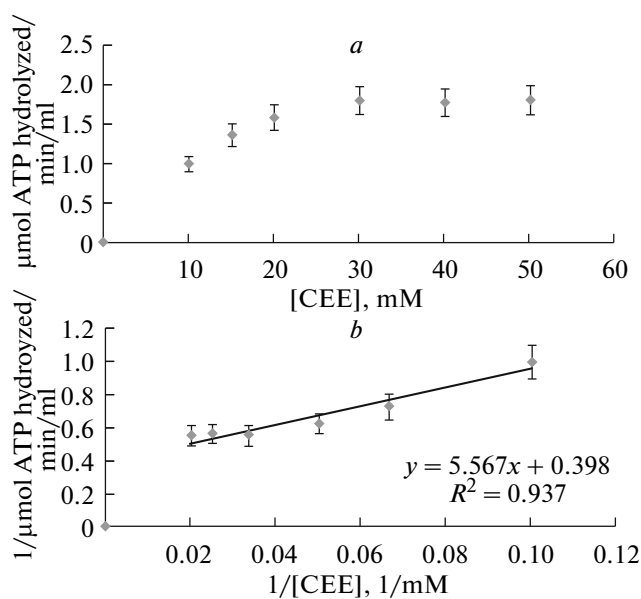

Fig. 2. Kinetic parameters of creatine kinase in the presence of creatine ethyl ester. *a* − The rate of ATP hydrolysis as a function of CEE concentration (10−50 mM). To calculate K_m and the relative V_{max} values for creatine ethyl ester, the Lineweaver−Burk plots is presented in panel *b*. Each point represents the mean ± SD of five different experiments.

perature dependence of CK activity in the presence of CEE, assayed at pH 7.5, showed 35°C to be optimal for CEE phosphorylation (fig. 4). The CK activity decreased by about 75% at temperatures above 40°C, and was absent above 60°C, indicating that the enzyme is not termostable. Below 35°C CK looses part
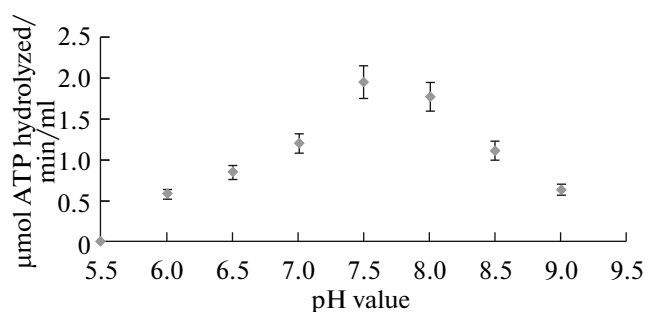
Fig. 3. pH-dependence of creatine kinase activity in the presence of 50 mM creatine ethyl ester. Enzymatic activity was measured in the pH range of 6–9 and expressed as IU/ml of the volume assay. Each point represents the mean ± SD of five different experiments.

Fig. 4. Temperature-dependence of creatine kinase activity in the presence of 50 mM creatine ethyl ester. Activity was measured within the temperature range of 20–75°C and expressed as IU/ml of the volume assay. Each point represents the mean ± SD of five different experiments.

of its activity: the decrement is about 50% at 25°C and 75% at 20°C.

DISCUSSION

The present biochemical study shows that CEE is a new substrate for CK, which can transform it into the phosphocreatine ethyl ester (PCEE), a new phosphagen compound (fig. 1*a, b*), even though its catalytic activity is lower than that observed with Cr (fig. 1*b*). Data are confirmed by a kinetic study (fig. 2), which also suggests that the conversion is due to the enzymatic activity. CK affinity for CEE (K_m 13.9 mM) was also lower than that for Cr ($K_m = 7$ mM). On the other hand, CK activity on creatinine was undetectable, suggesting that the result with CEE is not due to an aspecific action. Optimal pH and temperature values for the conversion of CEE into PCEE were estimated to be 7.5 and 35°C, respectively (fig. 3 and fig. 4). Overall, data suggested that CEE can be utilized by CK as a substrate, most likely in order to increase the phosphagens pool. CEE is presently used in several parapharmacologic dietary supplements to increase athletic performance or to improve the energetic status in pathological condition, however, it is considered to be a creatine source. It is tempting to presume that CEE is transformed to PCEE, contributing to the ATP balance, improving the phosphagen pool. The latter plays a fundamental role in tissues where energy demand is high, such as muscle, heart or brain. In fact, brain spends up to 20% of the body's energy, even though it represent only about 2% of body mass [22]. The role of these molecules on neuronal activity in healthy and pathological conditions is well known. During physiological function of neurons, an effective coupling of ATP-generating and ATP-consuming processes is needed to maintain a sufficiently high energy transfer since cellular processes are widely distributed and sites of high-energy consumption are often localized at re-
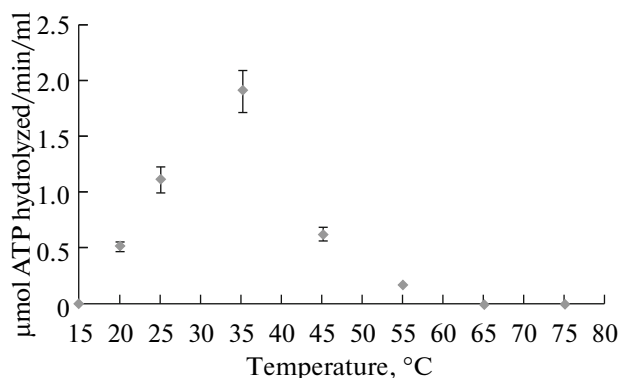
mote locations from the neuronal cell body, i.e., at the synapses [23]. For this reason, the CK/phosphagen-system is assumed to play a critical function in neuronal ATP metabolism [24] in the brain and spinal cord [25]. Cr supplementation may lead to improved functions of these systems. Indeed, positive effects of orally administered Cr on mental performance have been reported in healthy volunteers in a controlled double-blinded study [12]. In the brain PCr depletion is associated with disruption of neuronal functions, e.g., loss of hippocampal mossy fiber connection [25], and changes in the mitochondrial structure, showing intramitochondrial uMt-CK-rich inclusion bodies [26] that are typical for several clinical pathological conditions, such as encephalomyopathies and mitochondrial myopathies [12]. In several cases PCr loss determines mental retardation, speech delay, autism and even brain atrophy [27]. Diet integration with Cr was recently proposed to improve these pathological conditions. Recently, also CEE diet supplementation was shown to play a pivotal role in improving cognitive capacity [28, 29], what may depend on a direct effect of PCEE deriving from CK activity or on CEE conversion to Cr. However, it was recently reported that CEE can be cyclized into creatinine through a non-enzymatic reaction at physiological conditions encountered during its transit into the gastrointestinal tract. Since this causes a rise in the creatinine blood level [30, 31], this implies that elevated serum creatinine does not always indicate impaired renal function, but may depend on the use of CEE supplements [30]. Data reported herein suggests that CEE may increase cognitive capacity, not only because it is a Cr source, but also because it may become a substrate of CK which transforms it into PCEE, a new phosphagen molecule.

REFERENCES

1. Andres R.H., Ducray A.D., Schlattner U., Wallimann T., Widmer H.R. 2008. Functions and effects of creatine in the central nervous system. *Brain Res. Bull.* **76**, 329–343.

2. Brosnan M.E., Edison E.E., da Silva R., Brosnan J.T. 2007. New insights into creatine function and synthesis. *Adv. Enzyme Regul.* **47**, 252–260.

3. Braissant O., Henry H., Loup M., Eilers B., Bachmann C. 2001. Endogenous synthesis and transport of creatine in the rat brain: an *in situ* hybridization study. *Mol. Brain Res*. **86**, 193–201.

4. Braissant O., Henry H., Villard A.M., Speer O., Wallimann T., Bachmann C. 2005. Creatine synthesis and transport during rat embryogenesis: spatiotemporal expression of AGAT GAMT and CT1. *BMC Dev. Biol.* **5**, 9.

5. Peral M.J., Garcia-Delgado M., Calonge M.L., Duran J.M., de La Horra M.C., Wallimann T., Speer O., Ilundain A. 2002. Human, rat and chicken small intestinal Na - Cl -creatine transporter: functional, molecular characterization and localization. *J. Physiol.* **545**, 133–144.

6. cLeish M.J., Kenyon G.L. 2005. Relating structure to mechanism in creatine kinase. *Crit. Rev. Biochem. Mol. Biol*. **40**, 1–20.

7. Saks V.A., Ventura-Clapier R., Aliev M.K. 1996. Metabolic control and metabolic capacity: two aspects of creatine kinase functioning in the cells. *Biochim.Biophys.Acta*.**1274**, 81–88.

8. Wallimann T., Dolder M., Schlattner U., Eder M., Hornemann T., O'Gorman E., Ruck A., Brdiczka D. 1998. Some new aspects of creatine kinase (CK): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetics and physiology. *Biofactors*. **8**, 229–234.

9. Wyss M., Smeitink J., Wevers R.A., Wallimann T. 1992. Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. *Biochim. Biophys. Acta.* **1102**, 119–166.

10. Wallimann T., Schnyder T., Schlegel J., Wyss M., Wegmann G., Rossi A.M., Hemmer W., Eppenberger H.M., Quest A.F. 1989. Subcellular compartmentation of creatine kinase isoenzymes, regulation of CK and octameric structure ofmitochondrial CK: important aspects of the phosphoryl-creatine circuit. *Prog. Clin. Biol. Res.* **315**, 159–176.

11. Ventura-Clapier R., Kuznetsov A., Veksler V., Boehm E., Anflous K. 1998. Functional coupling of creatine kinases in muscles: species and tissue specificity. *Mol. Cell. Biochem.***184**, 231–247.

12. Wallimann T., Hemmer W. 1994. Creatine kinase in non-muscle tissues and cells. *Mol. Cell. Biochem.* **133–134**, 193–220.

13. Chen L., Roberts R., Friedman D.L. 1995. Expression of brain-type creatine kinase and ubiquitous mitochondrial creatine kinase in the fetal rat brain: evidence for a nuclear energy shuttle. *J. Comp. Neurol.* **363**, 389–401.

14. Meyer R.A., Sweeney H.L., Kushmerick M.J. 1984. A simple analysis of the "phosphocreatine shuttle". *Am. J. Physiol.* **246**, C365–C377.

15. Bessman S.P., Carpenter C.L. 1985. The creatine–creatine phosphate energy shuttle. *Annu. Rev. Biochem.* **54**, 831–862.

16. Wallimann T., Wyss M., Brdiczka D., Nicolay K., Eppenberger H.M. 1992. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem. J.* **281**, 21–40.

17. Schlattner U., Wallimann T. 2004. Metabolite channeling: creatine kinase icrocompartments. In: *Encyclopedia of biological chemistry*. EdsLennarz W.J., Lane M.D. N.Y.: Acad. Press, pp. 646–651.

18. Hespel P., Maughan R.J., Greenhaff P.L. 2006. Dietary supplements for football. *J. Sports Sci.* **24**, 749–761.

19. Tipton K.D., Ferrando A.A. 2008. Improving muscle mass: response of muscle metabolism to exercise, nutrition and anabolic agents. *Essays in Biochemistry.* **44**, 85–98.

20. Ferguson C., Shengli J. 2008. Method of preparing creatine ester salts of enhanced efficiency and bioavailability, and uses thereof for purposes of performance and lean mass enhancement. *US Pat. Appl. Publ.* 103202, 5.

21. Bergmeyer H.V. (Ed.). 1974. In: *Methods of Enzymatic Analysis*. Verlag Chemie-Acad. Press.

22. Ravera S., Panfoli I., Calzia D., Aluigi M.G., Bianchini P., Diaspro A., Mancardi G., Morelli A. 2009. Evidence for aerobic ATP synthesis in isolated myelin vesicles. *Int. J. Biochem. Cell. Biol.* **41**, 1581–1591.

23. Ames III A. 2000. CNS energy metabolism as related to function. *Brain Res. Rev.* **34**, 42–68.

24. Erecinska M., Silver I.A. 1989. ATP and brain function. *J. Cereb. Blood Flow Metab.* **9**, 2–19.

25. Watanabe A., Kato N., Kato T. 2002. Effects of creatine on mental fatigue and cerebral hemoglobin oxygenation. *Neurosci. Res.* **42**, 279–285.

26. In 't Zandt H.J., Renema W.K., Streijger F., Jost C., Klomp D.W., Oerlemans F., van der Zee C.E., Wieringa B., Heerschap A. 2004. Cerebral creatine kinase deficiency influences metabolite levels and morphology in the mouse brain: a quantitative *in vivo* 1H and 31P magnetic resonance study. *J. Neurochem.* **90**, 1321–1330.

27. Wyss M., Wallimann T. 1994. Creatine metabolism and the consequences of creatine depletion in muscle. *Mol. Cell. Biochem.***133–134**, 51–66.

28. Stockler S., Schulz P.W., Salomons G.S. 2007. Cerebral creatine deficiency syndromes: clinical aspects treatment and pathophysiology. In: *Creatine and creatine kinase in health and disease. Subcellular biochemistry*. Eds Salomons G.S., Wyss M. Dordrecht, The Netherlands: Springer, **46**, 149–166.

29. Ling J., Kritikos M., Tiplady B. 2009. Cognitive effects of creatine ethyl ester supplementation. *Behav. Pharmacol.* **20**, 673–679.

30. Velema M.S., de Ronde W. 2011. Elevated plasma creatinine due to creatine ethyl ester use. *Netherlands J. Med.* **69**, 79–81.

31. Giese M.W., Lecher C.S. 2009. Non-enzymatic cyclization of creatine ethyl ester to creatinine. *Biochem. Biophys. Res. Commun.* **388**, 252–255.